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Molecular Cloning and Characterization of a Human Eotaxin Receptor Expressed Selectively on Eosinophils

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Summary

The chemokine eotaxin is unusual in that it appears to be a highly specific chemoattractant for eosinophils. Ligand-binding studies with radiolabeled eotaxin demonstrated a receptor on eosinophils distinct from the known chemokine receptors CKR-1 and -2. The distinct eotaxin-binding site on human eosinophils also bound RANTES (regulated on activation T expressed and secreted) and monocyte chemotactic protein (MCP)3. We have now isolated a cDNA from eosinophils, termed CKR-3, with significant sequence similarity to other well characterized chemokine receptors. Cells transfected with CKR-3 cDNA bound radiolabeled eotaxin specifically and with high affinity, comparable to the binding affinity observed with eosinophils. This receptor also bound RANTES and MCP-3 with high affinity, but not other CC or CXC chemokines. Furthermore, receptor transfectants generated in a murine B cell lymphoma cell line migrated in transwell chemotaxis assays to eotaxin, RANTES, and MCP-3, but not to any other chemokines. A monoclonal antibody recognizing CKR-3 was used to show that eosinophils, but not other leukocyte types, expressed this receptor. This pattern of expression was confirmed by Northern blot with RNA from highly purified leukocyte subsets. The restricted expression of CKR-3 on eosinophils and the fidelity of eotaxin binding to CKR-3, provides a potential mechanism for the selective recruitment and migration of eosinophils within tissues.

Chemotactic cytokines, or chemokines, are 8–10-kD heparin-binding proteins that mediate a range of pro-inflammatory effects on leukocytes, such as chemotaxis, degranulation, synthesis of lipid mediators, and integrin activation (1–3). The chemokines are related in primary structure with amino acid sequence similarities ranging from 20 to 70% and are traditionally divided into two families, CXC (α chemokines) and CC (β chemokines), based on the presence or absence of an intervening amino acid in the first of two conserved cysteine pairs. CXC family members are primarily chemoattractants for neutrophils and include IL-8, neutrophil-activating protein (NAP)¹2, and platelet factor (PF4), whereas the CC family members, which include macrophage inflammatory protein (MIP)1 α , -1 β , RANTES (regulated on activation T expressed and se-

creted), monocyte chemotactic protein (MCP-1, MCP-2, MCP-3), and eotaxin, generally attract other leukocyte types such as monocytes, T cells, basophils, and eosinophils (1–5). More recently, a chemokine called lymphotactin with a single cysteine pair in the molecule has been identified that attracts lymphocytes (6).

The importance of chemokines in leukocyte trafficking has been demonstrated in several animal models. For example, neutralizing antibodies to IL-8 inhibit neutrophil recruitment to sites of inflammation such as endotoxin-induced pleurisy and reperfusion injury (7–9). Neutrophil recruitment is also impaired in IL-8 receptor knockout mice (10). MIP-1 α knockout mice were shown to have reduced inflammatory responses to viral infection (11) as demonstrated by a delay in T cell-dependent viral clearance of influenza, and elimination of coxsackie virus-mediated myocarditis. Furthermore, neutralizing antibodies to MIP-1 α were reported to influence eosinophil recruitment into mouse lung in a model of antigen-specific airway inflammation (12). Finally, antibodies to MCP-1 were able to block monocyte recruitment in a granuloma model (13) and to

¹Abbreviations used in this paper: CKR-1, chemokine receptor 1; IL-8RA, IL-8 receptor A; MCP-1, monocyte chemotactic protein 1; MIP-1 α , macrophage inflammatory protein 1 α ; NAP-2, neutrophil-activating protein 2; RANTES, regulated on activation T expressed and secreted; RT, reverse transcriptase; 7TMS, 7 transmembrane spanning.

completely inhibit T cell recruitment and cutaneous delayed-type hypersensitivity-induced inflammation in rats (14).

The only human CXC chemokine receptors characterized to date are the IL-8 receptor A, (IL-8RA) which binds IL-8, and the IL-8 receptor B (IL-8RB), which binds a number of CXC chemokines including IL-8 and GRO α (15–17). The most fully characterized CC chemokine receptors include CKR-1 which binds MIP-1 α and RANTES with high affinity (18, 19) and CKR-2, which binds MCP-1 with high affinity and MCP-3 with lower affinity (20). CKR-2 has been shown to exist in two isoforms resulting from the use of an alternative splice site in isoform A producing a distinct cytoplasmic tail. Isoform B, which is not spliced in this region, has been shown to be a functional receptor for MCP-1 and -3 in binding and signal transduction assays (20, 21). More recently, Power et al. (22) have reported a new receptor called CKR-4; cRNA from this receptor produces a Ca²⁺-activated chloride current in response to MCP-1, MIP-1 α , and RANTES when injected into *Xenopus laevis* oocytes. All are members of the 7 transmembrane-spanning (7TMS) G protein-coupled receptor superfamily (23, 24).

Eosinophils are selectively recruited into certain inflammatory lesions as a result of IgE-mediated reactions, for instance in rhinitis and allergic asthma, and also in response to certain parasitic infections (25–28). A number of chemoattractants have been identified for eosinophils, such as platelet-activating factor (29), C5a (30), IL-16 (31), RANTES, and MCP-3 (32–35), although these chemoattractants also induce the migration of other leukocyte cell types. The chemokine eotaxin, however, first identified in guinea pig (4) and subsequently in mouse (36) and human (5), is unusual in that it is selectively chemotactic for eosinophils. Binding studies with ¹²⁵I-labeled eotaxin and peripheral blood eosinophils strongly suggested that eotaxin binds a receptor on eosinophils distinct from CKR-1 and -2, as specific binding cannot be competed with MIP-1 α or MCP-1 (5). This finding would appear to eliminate CKR-4 as an eotaxin receptor as it is reported to signal with MIP-1 α and MCP-1. Based on ligand binding and receptor cross-desensitization data using eosinophils, it was predicted that at least one additional receptor exists that binds and signals in response to RANTES, MCP-3 (33, 35), and eotaxin (5), but not MIP-1 α .

Because of the strong correlation between eosinophil recruitment and tissue damage in allergic inflammatory diseases such as asthma (27, 37–40) and the probable role that eotaxin plays in such a response (4, 41–43), molecular characterization of the eotaxin receptor may assist in the development of receptor antagonists that may have therapeutic value. We report here the identification of a eosinophil-specific 7TMS G protein-coupled receptor that has significant sequence similarity to CKR-1 and -2. When expressed in a heterologous system, this receptor confers specific, high affinity binding of radiolabeled eotaxin, RANTES and MCP-3, as well as the capacity of the transfectants to migrate chemotactically to these ligands. Furthermore, an anti-CKR-3 mAb shows that it is expressed exclusively on the surface of eosinophils.

Materials and Methods

Cells, Cell Lines, and Tissue Culture. Eosinophils were isolated from heparinized blood using CD16 microbeads (Miltenyi Biotec, Auburn, CA), as described (5) and were shown cytologically to be $\geq 99\%$ pure. Neutrophils and PBMC were isolated as described (5). Monocytes were purified by CD14 positive selection with magnetic beads and T cells by passage of lymphocytes over nylon wool. To generate CD3 blasts, 2×10^6 PBMC/ml in RPMI-1640 plus 10% FCS were added to tissue culture plates first coated with the anti-CD3 antibody TR77. After 4–6 d, blasts were removed to fresh media and supplemented with IL-2 (Genzyme Corp., Cambridge, MA) at 50 U/ml.

Isolation of Genomic and cDNA Clones, Southern and Northern Hybridizations. Two independent approaches were taken to clone novel chemokine receptors from eosinophils: (a) reverse transcriptase (RT)-PCR with eosinophil mRNA and degenerate oligonucleotides based on known chemokine receptors, and (b) screening of a eosinophil cDNA library with the CKR-1 cDNA under low stringency wash conditions.

For the RT-PCR approach, 20 ng of eosinophil mRNA was reverse transcribed with oligo dT. 4 μ l of this cDNA was mixed with 200 μ M dNTP and 100 pmol of degenerate primers in a 50- μ l volume. Primer pairs TM2a-1 and TM3R were used in the first reaction in 60 mM Tris-HCl, pH 9.5, and 1.5 mM MgCl₂. The cycle parameters were 3 cycles: 94°C, 30 s; 37°C, 30 s; 2-min ramp to 72°C, 1 min, followed by 30 cycles: 94°C, 45 s; 48°C, 1 min; 72°C, 1 min. A second PCR was performed with a nested primer TM2a-2 and the TM3R primer with reaction conditions exactly as for the first round. The RT-PCR primers used to amplify receptor fragments from eosinophils were TM2a-1: 5'-TAC CTG CTS AAC CTG GCC ITG GCI G, which primes in a conserved region within the second TM spanning domain, and TM3R: 5'-GGC RTG GAC IAT GGC CAG GTA RCG GTC, which primes in a highly conserved region just 3' to the third TM spanning domain. The nested primer TM 2a-2: 5'-AC CTG GCC ITG GCI GAC CTM CTC TT corresponds to a region beginning 11 nucleotides 3' of the first nucleotide of TM2a-1. PCR products were assessed and separated by agarose gel electrophoresis. A fragment of predicted size (~200 bp) based upon known chemokine receptors was purified and subcloned into pCR-Script™ (Stratagene Inc., La Jolla, CA) for sequence analysis. This fragment was used to probe a human genomic library (Clontech, Palo Alto, CA) as described (5). One clone, EosL2, contained a 1.8-kb HindIII fragment predicted from Southern hybridizations that was subcloned into pBluescript II KS+ (Stratagene Inc.) for sequence analysis and further manipulation.

For isolation of cDNA clones, eosinophils were obtained from a patient diagnosed with idiopathic hyper-eosinophilic syndrome. RNA was isolated using a standard guanidinium isothiocyanate/cesium chloride method. mRNA was obtained using Dynabeads (Dynal, Inc., Great Neck, NY), and a bacteriophage library was constructed using the SuperScript™ Lambda System for cDNA synthesis and λ gt22A cloning arms (GIBCO BRL, Gaithersburg, MD). Approximately 750,000 PFU were screened with a full-length radiolabeled cDNA probe encoding CKR-1. The library was screened and phage isolated and purified using standard molecular biological techniques (44). Phage inserts were subcloned into pBluescript for sequence analysis.

Human genomic DNA was purchased from Clontech and Southern hybridization was as described (45). For Northern hybridization, RNA was isolated using TriZOL™ reagent (GIBCO BRL) following the manufacturer's recommended protocol. 15 μ g of total RNA was separated on 1.2% formaldehyde agarose gels and

transferred to Nytran-Plus™ nylon membrane (Schleicher & Schuell, Inc., Keene, NH) and cross-linked using a Stratalinker® (Stratagene Inc.). Hybridizations with radiolabeled probe was with ExpressHyb™ Solution (Clontech) using the manufacturer's suggested protocol. Length of autoradiograph exposure is described in appropriate figure legends. For hybridization, a 3' untranslated region probed specific for CKR-3 was used encompassing nucleotides 1203-1453 as deposited with EMBL/GenBank/DDBJ under accession number U49727.

Expression Vector Construction and Generation of a CKR-3-expressing Stable Cell Line. PCR was used to modify the CKR-3 gene contained in the 1.8-kb genomic fragment by inserting a HindIII restriction site and optimal Kozak sequence immediately 5' to the initiation codon. The coding region and 448 bp of 3' untranslated region were then cloned into the HindIII site of pcDNA3 (Invitrogen, San Diego, CA).

The murine pre-B lymphoma cell line L1.2 was obtained from Dr. Eugene Butcher (Stanford University, Stanford CA) and maintained in RPMI-1640 supplemented with 10% bovine serum. 20 µg of linearized, CKR-3 in pcDNA3 was used to transfect the cell line as follows. L1.2 cells were washed twice in HBSS and resuspended in 0.8 ml of the same. The plasmid DNA was mixed with the cells and incubated for 10 min at room temperature, transferred to a 0.4-cm electroporation cuvette, and a single pulse was then applied at 250 V, 960 µF. The electroporation was followed by a 10-min incubation at room temperature. G418^r was added to a final concentration of 0.8 mg/ml 48 h after transfection and the cells were plated in 96-well plates at 25,000 cells/well. After 2-3 wk under drug selection, G418-resistant cells were stained with 5H12 antireceptor mAb (see below) and analyzed by FACScan® (Becton Dickinson & Co., Mountain View, CA). Lines with detectable surface staining were expanded and cloned several times by limiting dilution. Clones with the brightest surface staining were further analyzed by Northern hybridization to confirm the presence of transfected receptor as well as by RT-PCR using a T7 primer present in the pcDNA 3 vector as the 5' primer and a CKR-3-specific primer as the 3' primer (not shown). No amplification was seen without addition of reverse transcriptase. For transient transfection, 20 µg of supercoiled DNA was used in the electroporation exactly as described for stable cell line production. Cell surface staining was assessed after 48-72 h. Transfected cells were treated with 5 mM *n*-butyric acid for 24 h before experimentation (46).

mAb Production and Flow Cytometry. mAbs reactive with CKR-3 were generated by immunizing mice with a synthetic peptide, corresponding to the NH₂-terminal 35 amino acids, coupled to the carrier protein PPD (Severn Biotech Ltd., Cambridge, UK). Female BALB/c mice were immunized with 50 µg of this peptide-PPD conjugate in PBS four times at 2-wk intervals. Mice were immunized intraperitoneally using CFA (first immunization) and IFA (subsequent injections). The final immunization was injected intravenously without adjuvant. 4 d later, the spleen was removed and cells were fused with the SP2/0 cell line as described (47). mAbs reactive with synthetic peptide were screened by ELISA as follows. 50 µl of peptide, at a concentration of 2 µg/ml in carbonate buffer, was used to coat 96-well Maxisorp plates for at least 4 h at 4°C (Nunc, Roskilde, Denmark). 300 µl/well of blocking buffer (PBS plus 1% BSA) was added for at least 2 h. Plates were washed four times with PBS/Tween 20, and 50 µl of mAb supernatant was added to each well and incubated at 37°C for 1 h. Plates were washed four times with PBS/Tween 20, and alkaline phosphatase-conjugated second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in

PBS was added to each well. After an incubation at 37°C for 30 min, plates were washed four times with PBS/Tween 20. The substrate used for the color reaction was *p*-nitrophenylphosphate dissolved in diethanolamine buffer (Bio-Rad Laboratories, Richmond, CA). Plates were read at 410 nm on an ELISA reader.

To determine which antipeptide mAbs could recognize native, surface-expressed CKR-3, the antipeptide mAbs were screened against transiently transfected cells and eosinophils. For mAb staining, cells were washed once with PBS, and resuspended in 100 µl PBS containing 2% FCS, 0.1% sodium azide (FACS® buffer), 5 µg/ml purified antibody, 5 µg/ml MOPC-21 IgG₁-isotype-matched control mAb (Sigma Chemical Co., St. Louis, MO), or 100 µl hybridoma culture supernatant. After 30 min at 4°C, cells were washed twice with FACS® buffer, and resuspended in 100 µl FITC-conjugated, affinity-purified F(ab')₂ goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). After incubating for 30 min at 4°C, cells were washed twice in FACS® buffer and analyzed by FACScan® to determine the level of surface expression. Propidium iodide was used to exclude dead cells.

Chemokines, Chemotaxis Assays, and Ligand-binding Assay. Recombinant human chemokines were obtained from Peprotech (Rocky Hill, NJ) except for eotaxin, which was synthesized using solid-phase methods that were optimized and adapted to a fully automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) as described (48). Chemotaxis experiments with L1.2 cells or L1.2 receptor transfectant cell lines were as described (5) except that endothelial cells were not used to coat the Bio-coat® transwell tissue culture inserts (Costar Corp., Cambridge, MA) and the incubation was for 4 h. Chemotaxis experiments with human eosinophils employed endothelial cells to coat the transwell inserts (5).

¹²⁵I-labeled eotaxin was produced using the Bolton Hunter reagent (NEN, Boston, MA), as described (47). The specific activity of radiolabeled eotaxin was calculated to be 180 Ci/mM. Chemokine binding to target cells was carried out using a modified method previously reported (49). Cells were washed once in PBS and resuspended in binding buffer (50 mM Hepes, pH 7.5, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA, and 0.05% azide) at a concentration of 10⁷/ml. Aliquots of 50 µl (5 × 10⁵ cells) were dispensed into microfuge tubes, followed by the addition of cold competitor and radiolabeled chemokines as indicated in the text. The final reaction volume was 200 µl. Nonspecific binding was determined by incubating cells with radiolabeled chemokines in the presence of 250-500 nM of unlabeled chemokines. After 60 min incubation at room temperature, the cells were washed three times with 1 ml of binding buffer containing 0.5 M NaCl. Cell pellets were then counted. The competition was presented as the percent specific binding as calculated by $100 \times [(S-B)/(T-B)]$, where *S* is the radioactivity of the sample, *B* is background binding, and *T* is total binding without competitors. Background binding was obtained by incubating cells with radiolabeled chemokine and at least 400-fold excess of unlabeled chemokines. Duplicates were used throughout the experiments and the standard deviations were always <10% of the mean. All experiments were repeated at least three times. Curve fit was calculated by Kaleidagraph software (Synergy Software, Reading, PA).

Results

Cloning of a CC Chemokine Receptor from Eosinophils. Two cloning approaches were taken, one using RT-PCR and degenerate oligonucleotides and the other crosshybridiza-

tion with a CKR-1 cDNA probe. Both approaches yielded the same sequence which had significant similarity to other 7TMS chemokine receptors. Both cDNA and genomic clones were sequenced and found to contain an open reading frame of 1,065 nucleotides encoding a protein of 355 amino acids with a predicted molecular mass of 41 kD (Fig. 1). Comparison with other chemokine receptor sequences

revealed a 62, 47, and 41% amino acid sequence similarity to CKR-1, -2B, and -4, respectively. In contrast, the amino acid sequence similarity to IL-8RA and IL-8RB was only 27% for both receptors.

The CKR-3 gene codes for four cysteine residues, one in each of the extracellular domains at AA positions 24, 106, 183, and 273. Cysteines at these positions are con-

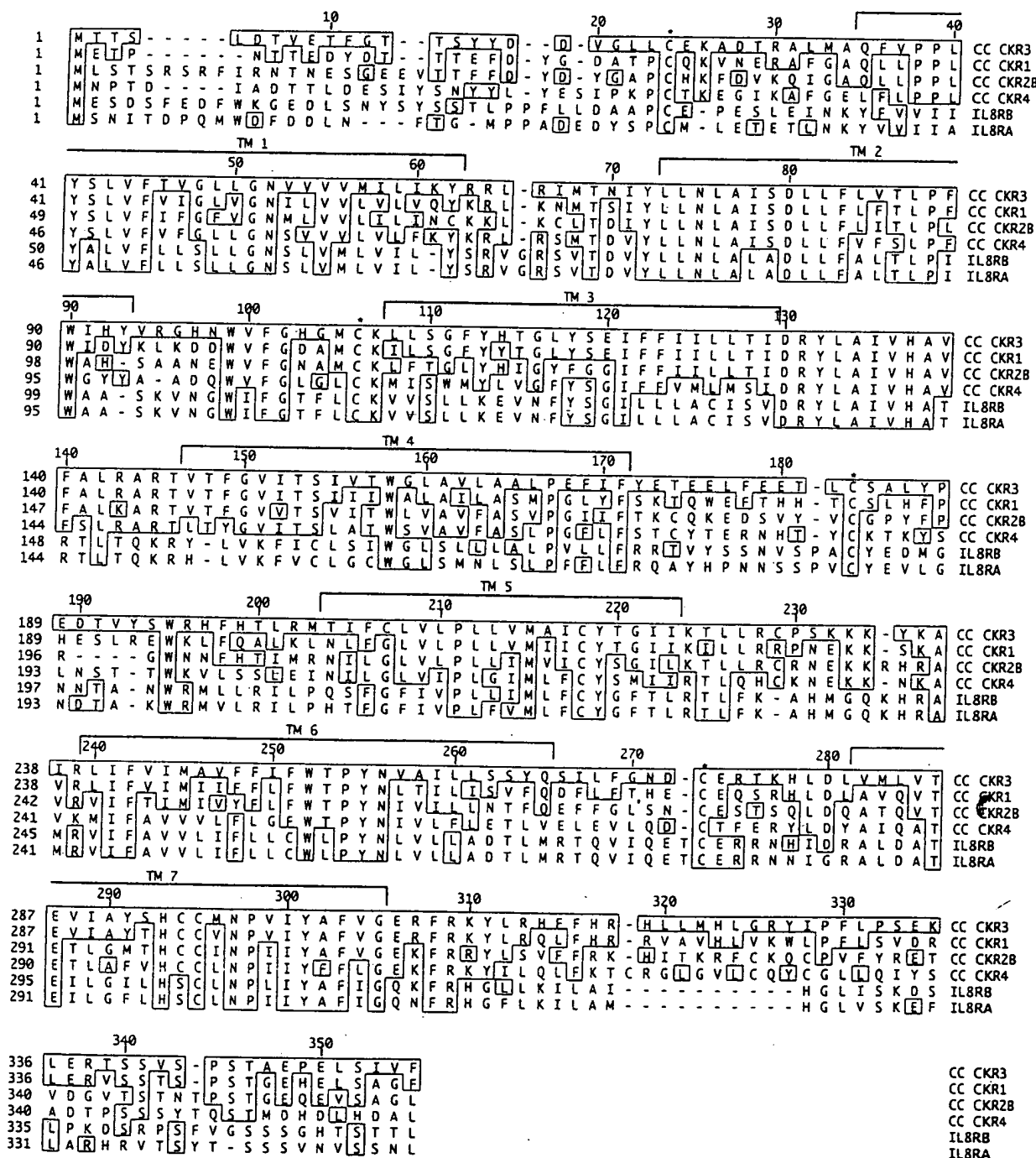


Figure 1. Amino acid sequence alignment of CKR-3 with other human chemokine receptors. The sequences were aligned by the Clustal method using MegAlign™ (DNASTAR, Inc., Madison, WI). Amino acids identical to CKR-3 are boxed with numbers across the top of the sequence referenced to CKR-3. (*) Four conserved extracellular cysteines. These sequence data are available from EMBL/GenBank/DBJ under accession number U49727.

served in all chemokine receptors (asterisks, Fig. 1). In addition, this receptor contains an amino acid motif, DRY-LAIVHA (amino acids 130-138, Fig. 1), which is also very highly conserved in all chemokine receptors and predicted to be intracellular. There are two consensus sites for protein kinase C phosphorylation (50, 51): one in the third intracellular loop at AA position 231, and one in the cytoplasmic tail at AA position 333. CKR-3 also contains eight serine/threonine residues in the cytoplasmic tail which may serve as phosphorylation sites for G protein-coupled receptor kinases such as those isolated from neutrophils (52) or other related family members (53, 54). Serine/threonine-rich cytoplasmic tails are also a common feature of chemokine receptors. Unlike CKR-1, -2, -4, IL-8RA, and IL-8RB receptors, CKR-3 does not contain sites for N-linked glycosylation.

The nucleic acid sequences obtained from genomic and cDNA libraries were colinear except for a region 17 bp 5' to the initiation codon. Here the genomic clone appears to have an intron that separates the promoter and most of the 5' untranslated region from the coding region. This genomic arrangement is similar in other 7TMS chemoattractant receptors (55, 56), including IL-8RA and IL-8RB (57-59) and CKR-1 (19). Furthermore, examination of the genomic sequence around the point of divergence reveals a canonical splice acceptor sequence (not shown).

We note that Combadiere et al. (60) have recently published a CKR-3-like sequence with specificity for MIP-1 α , -1 β , and RANTES. However, in light of the recent retraction of that data (61), further experiments will be necessary before comparison with the present work can be made.

Ligand Specificity of CKR-3. Attempts to express CKR-3 in traditional host cells such as COS, HEK 293, and CHO resulted in poor surface expression, yielding ambiguous ligand binding. Antibodies to a FLAG-tagged receptor (62) revealed that only 2-5% of the cells were surface positive whereas substantial intracellular protein could be detected, indicating a problem with protein trafficking. Therefore, to assess ligand-binding specificity and signal transduction of CKR-3, the clone was transfected into the mouse pre-B cell line L1.2 as an alternative host cell line in which high levels of surface expression could be more easily selected. This cell line has been used successfully for the study of other chemoattractant receptors (63), and the expression of transfected human chemokine receptors confers specific chemotactic ability to various ligands (see below). To monitor surface expression of CKR-3, a mAb was produced to the NH₂-terminal region of the receptor, by immunizing mice with a 35-amino acid, NH₂-terminal synthetic peptide. Anti-peptide mAbs were detected by ELISA, and mAbs that recognize the native receptor were identified by their reactivity with human eosinophils, as well as by their staining of transient transfectants. Fig. 2 A shows detectable surface staining of the transiently transfected receptor on a subpopulation of L1.2 cells, using an antireceptor mAb, 5H12. Untransfected L1.2 cells were negative (Fig. 2 B). Because of the low levels of surface expression in transient transfections, a stable cell line was constructed by limiting dilution

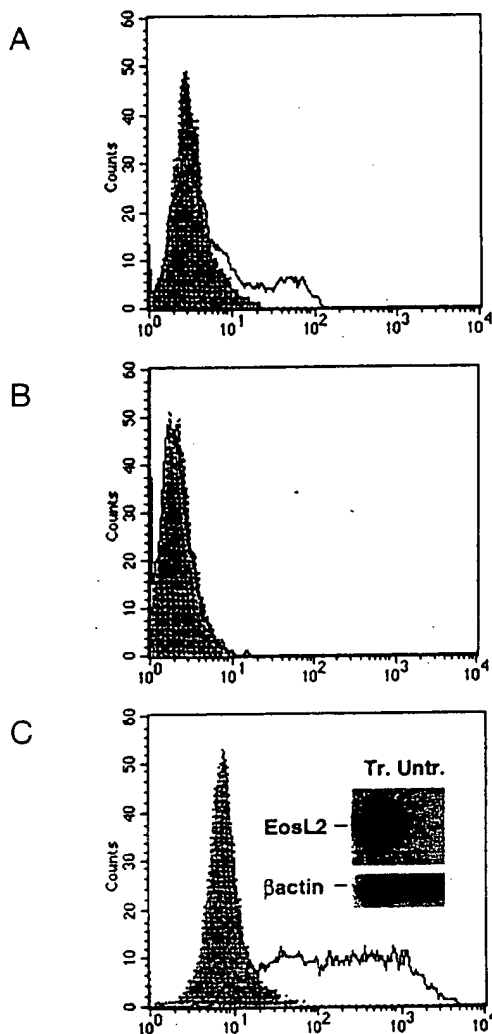


Figure 2. Cell surface staining of L1.2 cells and receptor transfectants with anti-CKR-3 mAb, 5H12. L1.2 cells transiently transfected with CKR-3 (A), L1.2 mock-transfected control cells (B), and the stable CKR-3 transfectant cell line, E5 (C) were stained as described in Materials and Methods. (Shaded plot) Background staining with the IgG₁-isotype-matched control mAb, MOPC-21. (Solid line) Staining with the antireceptor mAb, 5H12.

cloning of the transfectants and selecting for high surface staining. This yielded lines that had much higher levels of receptor expression (Fig. 2 C). Northern blot analysis confirmed the presence of transfected CKR-3 mRNA in one of the subclones, designated E5, and its absence in untransfected L1.2 cells (Fig. 2 C, inset).

The E5 cell line was tested for its ability to bind radiolabeled eotaxin. Fig. 3 A shows that the transfected cells bound ¹²⁵I-labeled eotaxin specifically and with high affinity. Scatchard analysis of the binding data indicated a dissociation constant (K_d) of 1.5 nM, similar to the value of 0.5 nM obtained using purified human eosinophils (Fig. 3 B). In addition, both RANTES and MCP-3 were able to specifically compete for binding. No other chemokines tested, including MIP-1 α , -1 β , or IL-8, were able to specifically compete for radiolabeled ligand (Fig. 4).

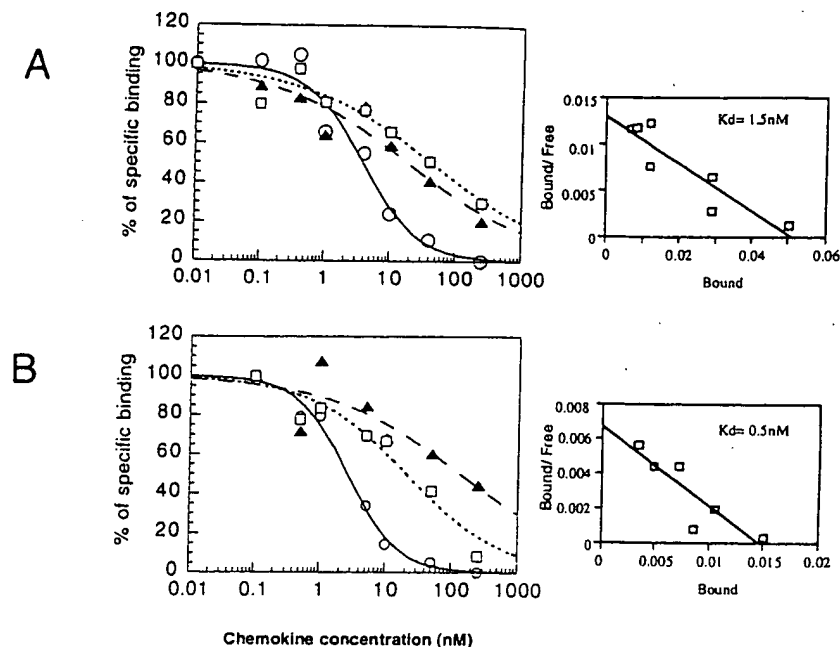


Figure 3. Competitive ligand binding of radiolabeled human eotaxin to L1-2/CKR-3 transfectant (A) and human eosinophils (B). Cells were incubated with 0.6 nM ^{125}I -labeled eotaxin and various concentrations of unlabeled eotaxin (O), RANTES (▲), and MCP-3 (□). After 60 min at room temperature, cell pellets were washed and counted. The total binding of eotaxin to CKR-3 transfectants was $11,611 \pm 119$ cpm and background binding was $2,248 \pm 745$ cpm. The total binding of eotaxin to eosinophils was $7,866 \pm 353$ cpm and background binding $1,148 \pm 518$ cpm. Scatchard plot of unlabeled eotaxin competition was calculated from the data and presented on the right of the binding curves.

CKR-3 Expression in L1.2 Cells Confers Chemotactic Ability to Eotaxin, RANTES, and MCP-3. To further investigate the ligand specificity of CKR-3, the L1.2 receptor transfectants were tested for their ability to migrate in response to a panel of chemokines over a range of doses (Fig. 5). The CKR-3-expressing cell line showed a chemotactic response to eotaxin, RANTES, and MCP-3 with a peak response to eotaxin at 100 ng/ml, although specific migration could be detected as low as 10 ng/ml (Fig. 5 A). Whereas a response to RANTES was evident at both 10 and 100 ng/ml, the magnitude of the response was not as great as with eotaxin. MCP-3 appeared to be a less potent chemoattractant on the transfected cell line than on eosinophils (5, 33; see below) with no detectable migration below 100 ng/ml. No significant response to other chemokines tested was seen

with this cell line. Furthermore, in other control experiments, cells did not migrate to the bottom chamber when chemokine was added to the top well alone, confirming that cell migration was chemotactic rather than chemokinetic (not shown). Fig. 5 B shows that the untransfected L1.2 cell line did not migrate in response to any chemokines tested. Indeed, a striking feature of the L1.2 cell line was the very low background chemotaxis to nonspecific ligands. As a specificity control, L1.2 cells transfected with IL-8RB migrated specifically to IL-8 and GRO α (Fig. 5 C) as well as to NAP-2 and ENA-78 (not shown), but not to other CXC or CC chemokines. Other chemokine receptors we have transfected into L1.2 cells also confer chemotactic ability to their specific ligands, including CKR-2 transfectants to MCP-1 and -3, CKR-1 transfectants to MIP-1 α , and IL-8RA transfectants to IL-8 (not shown). Pertussis toxin completely abrogated the chemotactic response of both eosinophils and the CKR-3 transfectants to eotaxin, indicating that the receptor was signaling through the G α subclass (64) in both normal and transfected cells (not shown).

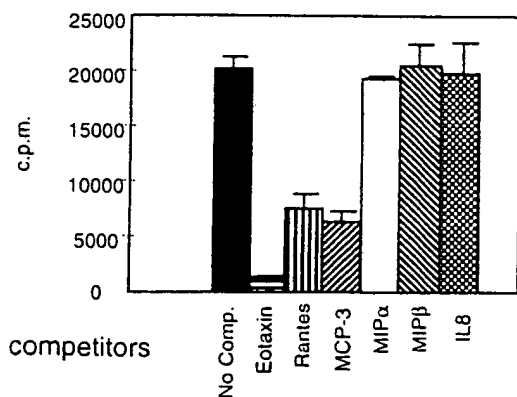


Figure 4. Inhibition of eotaxin binding by various chemokines. L1-2/CKR-3 transfectants were incubated with 0.6 nM radiolabeled eotaxin and 250 nM unlabeled chemokines or no competitor as indicated.

The Chemotactic Profile of Eosinophils Resembles that of CKR-3 Transfectants. To assess whether the function of normal eosinophils resembled that of CKR-3 L1.2 transfectants, chemotaxis experiments were performed using eosinophils from normal individuals with high levels of eosinophils (~6–8% of white blood cells). Fig. 6 shows two characteristic patterns of eosinophil chemotaxis observed in two different individuals. One pattern was characterized by a robust migration to eotaxin, and a lesser response to RANTES and MCP-3 (Fig. 6 A). The other pattern showed essentially equivalent chemotaxis to eotaxin, RANTES, and MCP-3. These patterns were not due to variations in the

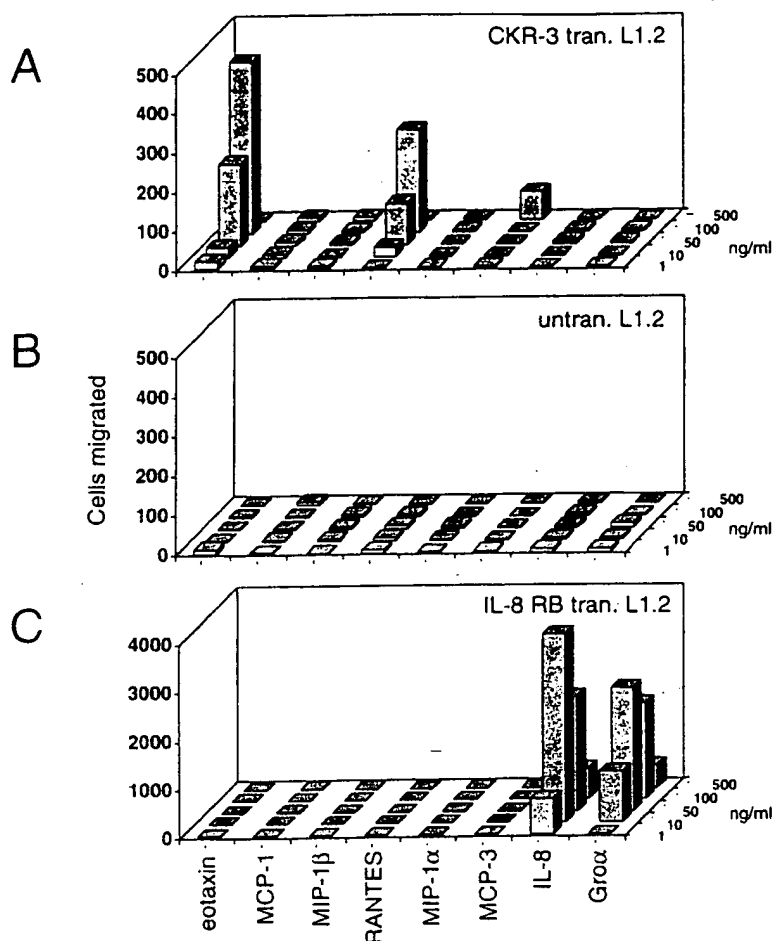


Figure 5. Transwell chemotaxis of the L1.2 cell line and L1.2 receptor transfectants. 10^6 cells of the CKR-3 transfected cell line E5 (A), the parental L1.2 cell line (B), and the IL-8 RB L1.2 receptor transfectant line LSLW-2 (C) were placed in the top chamber and chemokines were placed in the bottom chamber at the concentrations specified. Migration was allowed for 4 h and cells migrating to the bottom chamber were counted as described in Materials and Methods. All assays were performed in duplicate and the results are representative of at least three separate experiments. Chemokines are listed along the x-axis, number of cells migrated (as described in Materials and Methods) along the y-axis, and concentration of chemokine along the z-axis.

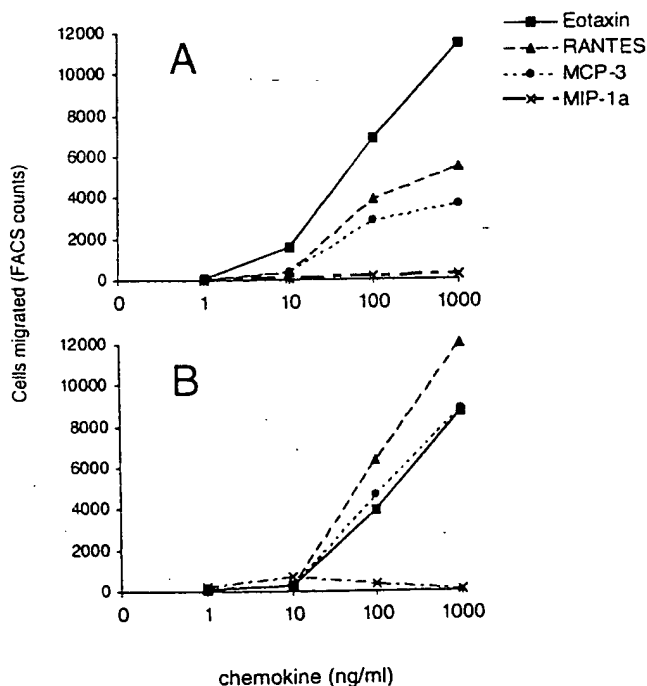


Figure 6. The chemotactic response of eosinophils from some individuals resembles that of CKR-3 L1.2 transfectants. Donor to donor variation of chemotactic responses of eosinophils to eotaxin, RANTES, MCP-3,

and MIP-1α was observed. Eosinophils were purified from blood and assessed for their chemotactic response to various concentrations of chemokines as described in Materials and Methods. Values are from a representative experiment of at least four performed, using the same two blood donors.

CKR-3 Is Expressed Selectively on Eosinophils. Although eotaxin is thought to be a specific chemoattractant for eosinophils, CKR-3 also binds RANTES and MCP-3, which are known to attract monocytes and T cells. Therefore, message expression of the receptor was examined in various leukocyte populations. Because of the high sequence similarity of CKR-3 to other CC chemokine receptors and the fact that the full-length clone hybridizes to multiple sequences in Southern blots, we selected as a probe a 3' untranslated region fragment that does not crosshybridize with other sequences in Southern blots (Fig. 7 A). Fig. 7 B shows a Northern blot panel of leukocyte populations including monocytes, neutrophils, lymphocytes, T cells, T cell blasts produced by activation with CD3 mAb, and eosinophils. The only cell population that gave a detectable signal was eosinophils, where a message 1.8 kb in size was found.

and MIP-1α was observed. Eosinophils were purified from blood and assessed for their chemotactic response to various concentrations of chemokines as described in Materials and Methods. Values are from a representative experiment of at least four performed, using the same two blood donors.

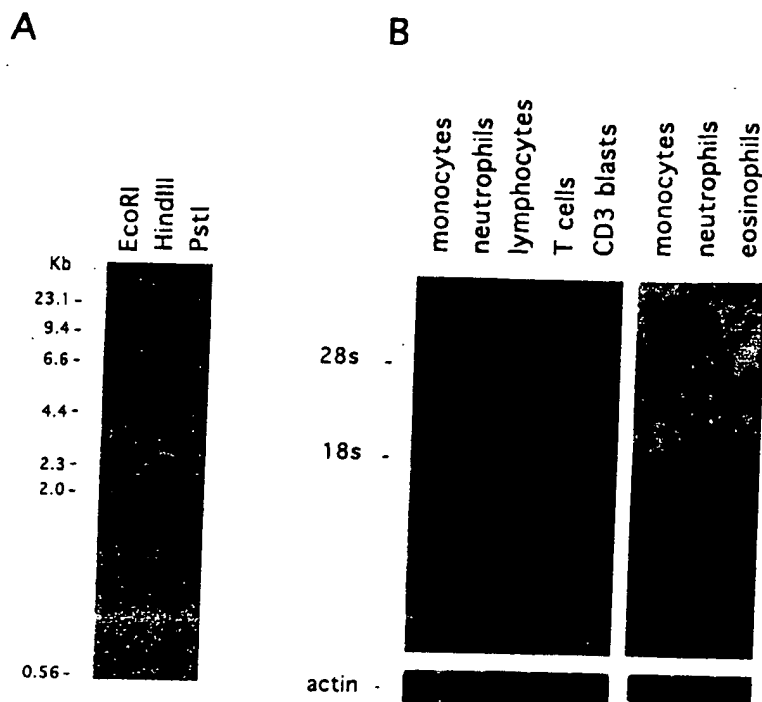


Figure 7. Southern and Northern hybridization analysis of CKR-3. (A) 10 μ g of human genomic DNA digested with restriction enzymes indicated was probed with a 250-bp DNA fragment from the 3' untranslated region of the CKR-3 gene. The autoradiograph was exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) for 48 h with intensifying screen. Size markers are indicated on the left. (B) 15 μ g of total RNA isolated from highly purified leukocyte populations was blotted onto Nytran-PlusTM membrane and probed with the same 3' untranslated region. Northern blots were exposed to X-OMAT AR film for 5 d with intensifying screen. Ribosomal RNA bands are indicated on the left. CKR-3-specific probe was removed by boiling in 0.5% SDS and the blot re-probed with β -actin to control for variation in loading.

To examine surface expression of CKR-3, various leukocyte types were stained with the mAb 5H12 and analyzed by flow cytometry. Highly purified eosinophils stained strongly with 5H12 (Fig. 8), suggesting abundant

expression of the receptor on the surface of eosinophils. This is consistent with the high receptor number determined by ligand binding and Scatchard analysis (5). Neutrophils, blood T cells, and monocytes showed little or no staining with this mAb (Fig. 8).

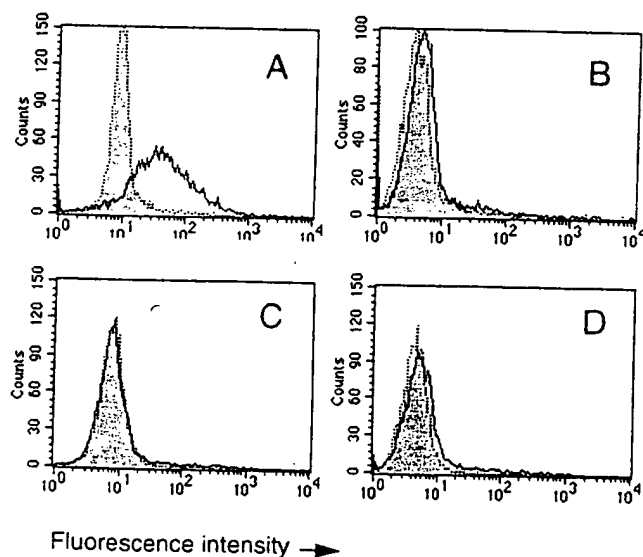


Figure 8. Examination of CKR-3 expression on leukocytes, using mAb 5H12 and flow cytometry. Leukocyte subsets were prepared as described in Materials and Methods, and stained with anti-CKR-3 mAb 5H12 (solid lines) or MOPC-21 control antibody (shaded profile). (A) eosinophils; (B) T cells; (C) monocytes; and (D) neutrophils. Dead cells were excluded based on propidium iodide staining. Staining profiles were representative of at least four experiments. T cells were identified based on CD3 staining. Monocytes and neutrophils were identified by forward and side scatter.

Discussion

In recent years, a novel family of G protein-coupled receptors has been cloned and characterized (Table 1); all of the receptors show a high degree of relatedness (Fig. 1) and share as ligands various members of the chemokine family. These receptors transduce a range of physiologic responses by host cells, from Ca^{2+} flux and granule release to integrin expression, chemotaxis, and most recently, inhibition of HIV replication (65). Their initial characterization on leukocytes has prompted the observation that they participate in host-immune responses; chemokine neutralization; targeted gene disruption studies support this concept. One

Table 1. Chemokine Receptor Profiles

Receptor	Chemokines that bind and signal	Reference
IL-8RA	IL-8	17
IL-8RB	IL-8, GRO α/β , NAP-2, ENA-78	15
CKR-1	MIP-1 α , RANTES, MCP-3	18, 19
CKR-2	MCP-1, MCP-3	20, 21
CKR-3	Eotaxin, RANTES, MCP-3	This report
CKR-4	MIP-1 α , RANTES, MCP-1	22
CKR-5	MIP-1 α , MIP-1 β , RANTES	76

characteristic of these receptors is their ability to bind several chemokines. In addition, the chemokines themselves usually bind more than one receptor. Less clear, however, is the distribution of these receptors among the various leukocyte classes. The eosinophil has attracted our attention because of its importance in certain pathologies and because of the uncertainty as to which receptor(s) is responsible for its selective recruitment.

A number of studies have demonstrated strong chemotactic responses by eosinophils to RANTES, MCP-3, and lately, eotaxin (5). The receptor on eosinophils that was responsible for this chemotaxis was originally thought to be CKR-1, the MIP-1 α /RANTES receptor, but intracellular calcium ($[Ca^{2+}]_i$) desensitization and ligand-binding studies implicated the existence of a distinct eosinophil receptor (5, 33, 35). We have cloned and characterized a receptor we call CKR-3 whose characteristics match those predicted for a eosinophil receptor. When transfected into L1.2 cells, CKR-3 binds eotaxin, RANTES, and MCP-3, but not MIP-1 α and confers chemotactic responses to eotaxin, RANTES, and MCP-3. CKR-3 is expressed on the surface of eosinophils at high levels, as revealed by mAb staining, and it is absent from other leukocyte types.

The ligand-binding experiments using CKR-3 transfectants indicate that CKR-3 is a high affinity receptor for eotaxin, and also recognizes RANTES and MCP-3. The chemotaxis of CKR-3 L1.2 transfectants was consistent with this binding pattern, since CKR-3 transfectants migrated in accord with the binding affinities of the chemokines. The ligand binding and chemotactic properties of the CKR-3 L1.2 transfectants resembled the ligand binding and chemotactic properties of human eosinophils. This would suggest that CKR-3 is one of the relevant receptors for normal eosinophil chemotactic responses, although we do not discount the possibility that other CC chemokine receptors may play a role. Eotaxin could compete effectively for all of the RANTES and MCP-3 binding sites on human eosinophils (5), suggesting that most if not all of the RANTES and MCP-3 activity on human eosinophils is through a eotaxin receptor. Nevertheless, human eosinophils from some individuals may express a small number of other receptors, since eosinophils from these donors show a small degree of $[Ca^{2+}]_i$ flux and/or chemotactic responses to MIP-1 α (reference 5 and Fig. 7). The role of MIP-1 α as a eosinophil chemoattractant has been controversial; some investigators detect chemotactic responses (32), whereas others do not (5, 66). Interestingly, MIP-1 α is a strong eosinophil chemoattractant in the mouse, and this appears to be mediated through the murine CKR-3 homologue, which also binds and signals with murine eotaxin (Gerard, C. and J.-C. Gutierrez-Ramos, manuscript in preparation).

The dose-response profile for chemokine-stimulated chemotaxis of the L1.2 transfectants matches the eosinophil profile in qualitative, but not quantitative terms. This is particularly seen in the case of MCP-3, which is a potent agent on eosinophils but not L1.2 transfectants. This contrasts with the IL-8R transfectants, which are very responsive to IL-8. Although complex, the preponderance of evi-

dence now favors the G protein activation of PLC, with subsequent release of inositol phosphates and $[Ca^{2+}]_i$ as the initial steps in leukocyte locomotion (67, 68). The lack of potency of CKR-3 in promoting chemotaxis may reflect a lack of efficient coupling between the receptor, G protein subunits, and the PLC isoform in these cells. In agreement with this is the observation that the IL-8R-transfected L1.2 cells demonstrate a vigorous calcium flux in response to IL-8, whereas there is a weak signal, if any, detected for CKR-3-stimulated cells.

In granulocytes, the PLC isoform β 2 is activated by the β/γ G protein subunits (69). Other PLC isoforms are activated by the α subunits (70). Since the IL-8R and CKR-3 transfectants are pertussis toxin sensitive, we may conclude that each receptor is coupled to an α i subunit. However, the identities of the PLC isoform and the β/γ subunits are unknown. In future experiments, the L1.2 cells may provide an interesting system to study the role of β/γ subunits and the PLC in receptor coupling to chemotaxis via CKR-3.

The CKR-3 has several features that distinguish it from other CC chemokine receptors identified to date. First, it is expressed at a high level, $\sim 40,000$ – $50,000$ sites per cell, compared to CKR-1 and -2 which are usually expressed on monocytes and T cells at $< 3,000$ sites per cell (71, 72). Neutrophils express $\sim 40,000$ – $60,000$ IL-8R per cell (73), suggesting that cells of the granulocytic lineage generally express high levels of chemokine receptors. Neutrophils and eosinophils are two of the most responsive cell types in chemotaxis assays, although whether this relates to receptor levels is uncertain. The second unusual feature of CKR-3 is its restricted expression, since most chemokine receptors are expressed on a number of leukocyte types. Although we have not detected message in resting or activated T cells, we cannot rule out the possibility that a minor subset of T cells may express this receptor. Restricted expression of CKR-3 on eosinophils may correlate with the need for highly selective recruitment of these cells, for instance, in response to certain parasitic infections (25–27) and also in some inflammatory settings, such as asthma (26, 28). This selective recruitment presumably relates to the specific functions of eosinophils, such as killing of parasites, although their role in airway inflammation is not fully resolved. Another unusual feature of CKR-3 is the lack of an NH_2 -terminal N-linked glycosylation site, which is a conserved feature of many of the 7TMS receptors, although murine CKR-3 and -1 also lack this site (74).

The eotaxin-CKR-3 interaction provides a mechanism for the selective recruitment of eosinophils. This could be achieved at two levels: first, the CKR-3 is highly restricted in its expression to eosinophils. Second, eotaxin binds to and signals through this receptor with a high degree of fidelity, unlike, for instance, MCP-3 which binds CKR-1, -2 (75), and -3 (this report), and MIP-1 α which binds CKR-1 and -4 (18, 22). Indeed, eotaxin is possibly the only example of a chemokine that functions through a single receptor. Thus the production of eotaxin within a tissue might be expected to lead to selective eosinophil recruitment, and in support of this notion, we have observed that eotaxin in-

jection into the skin of rhesus monkeys leads to selective eosinophil migration (5). In these in vivo experiments, eotaxin was shown to recruit eosinophils at a 10-fold lower dose than RANTES, similar to the in vitro chemotaxis of CKR-3 transfectants. In addition, at high doses RANTES clearly recruits mononuclear cells to skin sites (5), an observation not made with eotaxin.

In conclusion, we have identified an important chemokine receptor on human eosinophils that appears to be the

primary receptor for eotaxin, RANTES, and MCP-3 binding, and also for the chemotactic responses of eosinophils to these three chemokines. The discovery of this receptor, and identification of its counter ligands, provide a basis for understanding the nature and circumstances of eosinophil recruitment to inflammatory sites. Disrupting eotaxin-CKR-3 interaction may prevent eosinophil recruitment to tissues, similar to the way in which IL-8R disruption inhibits neutrophil migration to certain inflammatory sites (9, 10).

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